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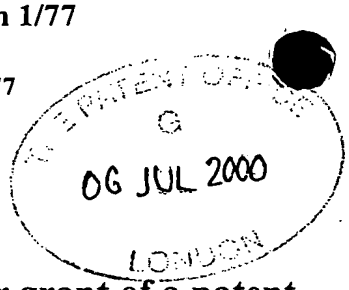

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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

SmithKline Beecham Biologicals s.a.
Rue de l'Institut 89, B-1330 Rixensart, , Belgium

Patents ADP number *(if you know it)* 75 33243001

If the applicant is a corporate body, give the country/state of its incorporation — Belgian

4. Title of the invention — Novel Vaccine

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Novel Vaccine

This invention relates to novel influenza vaccine formulations, methods for preparing them and their use in prophylaxis or therapy. In particular the invention relates to vaccines for administration to the mucosa, more particularly for nasal administration.

Influenza virus is one of the most ubiquitous viruses present in the world, affecting both humans and livestock. The economic impact of influenza is significant.

The influenza virus is an RNA enveloped virus with a particle size of about 125 nm in diameter. It consists basically of an internal nucleocapsid or core of ribonucleic acid (RNA) associated with nucleoprotein, surrounded by a viral envelope with a lipid bilayer structure and external glycoproteins. The inner layer of the viral envelope is composed predominantly of matrix proteins and the outer layer mostly of the host-derived lipid material. The surface glycoproteins neuraminidase (NA) and haemagglutinin (HA) appear as spikes, 10 to 12 nm long, at the surface of the particles. It is these surface proteins, particularly the haemagglutinin, that determine the antigenic specificity of the influenza subtypes.

Typical influenza epidemics cause increases in incidence of pneumonia and lower respiratory disease as witnessed by increased rates of hospitalisation or mortality. The elderly or those with underlying chronic diseases are most likely to experience such complications, but young infants also may suffer severe disease. These groups in particular therefore need to be protected.

Currently available influenza vaccines include whole inactivated virus vaccines, split particle vaccines, and subunit vaccines. Influenza vaccines, of all kinds, are usually trivalent vaccines. They generally contain antigens derived from two influenza A virus strains and one influenza B strain. A standard 0.5 ml injectable dose in most cases contains 15 μg of haemagglutinin antigen component from each strain, as measured by single radial immunodiffusion (SRD) (J.M. Wood et al.: An improved single radial immunodiffusion technique for the assay of influenza haemagglutinin antigen:

adaptation for potency determination of inactivated whole virus and subunit vaccines. J. Biol. Stand. 5 (1977) 237-247; J. M. Wood et al., International collaborative study of single radial diffusion and immunoelectrophoresis techniques for the assay of haemagglutinin antigen of influenza virus. J. Biol. Stand. 9 (1981) 317-330.

The influenza virus strains to be incorporated into influenza vaccine each season are determined by the World Health Organisation in collaboration with national health authorities and vaccine manufacturers.

Current efforts to control the morbidity and mortality associated with yearly epidemics of influenza are based on the use of intramuscularly administered inactivated influenza vaccines. The efficacy of such vaccines in preventing respiratory disease and influenza complications ranges from 75% in healthy adults to less than 50% in the elderly.

Influenza viruses, like many pathogens, invade at mucosal surfaces, initially in the upper respiratory tract. Mucosal immunity constitutes the first line of defence for the host and is a major component of the immune response in the nasal passages and in the airways of the lower respiratory tract. Although the presently used injectable influenza vaccines stimulate serum HA-specific IgG in the majority of healthy individuals, a significant rise in HA-specific nasal IgA antibody occurs in only a minority of vaccinated subjects. Improved influenza vaccines with better immunogenicity and clinical efficacy need to target both local and systemic antibody responses.

Experimental intranasal exposure of humans to inactivated influenza vaccines dates back as far as the 1940s (see review in Eyles et al. 2000 BioDrugs 13(1): 35-59). Although there was a resurgence of interest in the use of inactivated virus for IN immunisation in the 1960s and 70s, most attention in the intranasal field has been directed to the live attenuated approach.

Intranasally administered, live attenuated influenza vaccines for example cold-adapted vaccines offer improved mucosal immunity, with promising results particularly in children. However, this approach has failed so far to gain acceptance worldwide.

Thus, most of the commercially available influenza vaccines are either split or subunit injectable vaccines. These vaccines are prepared by disrupting the virus particle, generally with a surfactant, and separating or purifying the viral proteins to varying extents. Split vaccines are prepared by fragmentation of whole influenza virus, either infectious or inactivated, with solubilizing concentrations of surfactants and subsequent removal of the surfactant and some or most of the viral lipid material. Split vaccines generally contain contaminating matrix protein and nucleoprotein and sometimes lipid, as well as the membrane envelope proteins. Split vaccines will usually contain most or all of the virus structural proteins although not necessarily in the same proportions as they occur in the whole virus. Subunit vaccines on the other hand consist essentially of highly purified viral surface proteins, haemagglutinin and neuraminidase, which are the surface proteins responsible for eliciting the desired virus neutralising antibodies upon vaccination.

More recently, more highly purified, better characterised split influenza vaccines have been combined with adjuvants in an attempt to improve on the immunogenicity in adults and older people. In spite of significant increases in immune responses in mice, a number of approaches using new generation adjuvants have not proved possible to confirm in man.

Standards are applied internationally to measure the efficacy of influenza vaccines. The European Union official requirements for an effective vaccine against influenza are set out in the table below. The requirements are different for adult populations (18-60 years) and elderly populations (>60 years).

	18 - 60 years	> 60 years
Seroconversion rate*	>40%	>30%
Conversion factor**	>2.5	>2.0
Protection rate***	>70%	>60%

* Seroconversion rate is defined as the percentage of vaccinees who have at least a 4-fold increase in serum haemagglutinin inhibition (HI) titres after vaccination, for each vaccine strain.

** Conversion factor is defined as the fold increase in serum HI geometric mean titres (GMTs) after vaccination, for each vaccine strain.

*** Protection rate is defined as the percentage of vaccinees with a serum HI titre equal to or greater than 1:40 after vaccination (for each vaccine strain) and is normally accepted as indicating protection.

For an intranasal flu vaccine to be commercially useful it will not only need to meet those standards, but also in practice it will need to be at least as efficacious as the currently available injectable vaccines. It will also need to be commercially viable in terms of the amount of antigen and the number of administrations required.

Intranasal flu vaccines based on inactivated virus that have been studied over the past few decades have not met these criteria.

Fulk et al. 1969 (J. Immunol. 102, 1102-5) compared intranasal administration (nose drops plus nebulisation) of killed influenza virus with subcutaneous (s.c.) administration in elderly patients. Whereas 56% of the patients receiving s.c. administration exhibited a 4-fold increase in antibody titres (HI), the corresponding increase was observed in only 25% of those receiving an intranasal administration. Two intranasal administrations resulted in a 75% seroconversion.

Gluck et al. 1999 (J. Virol. 73, 7780-6) demonstrated that two consecutive intranasal inoculations administered by a spray containing potent mucosal adjuvants (*E. Coli* Heat Labile Toxin, HLT) were required to induce seroconversion (4-fold increase in humoral antibody response) comparable to an intramuscular administration. A single intranasal administration of 15 µg HA per strain in the presence of adjuvant, or even two administrations in the absence of adjuvant were incapable of providing equivalent seroconversion. The influenza antigen was in the form of virosomes, reconstituted

lipid bilayers produced using phosphatidylcholine and virus surface proteins extracted from egg-derived influenza virus.

The concept of using two or more intranasal administrations in order to attempt to achieve higher levels of seroconversion has also been used by other investigators. Petrescu et al. (1979. Rev. Rom. Med-Virol. 30, 109-115) administered inactivated virus (1000 international units per vaccination dose) intranasally twice over a two week period. Oh et al. (1992 Vaccine 10, 506-11) administered split vaccine in the form of a nasal spray four times at weekly intervals, 15 μg HA per strain each administration (0.25 ml per nostril on each occasion). Kuno-Sakai et al. (1994. Vaccine 12,1303-1310) administered, twice at an interval of 1 week, aerosol inactivated vaccine threefold the strength of commercially available split influenza. Recently Muszkat et al. (2000 Vaccine 18, 1696-9) administered two intransal immunisations with inactivated flu antigen (15 μg per dose) to elderly patients and observed higher systemic seroconversion via nasal immunisation to that obtained by injection.

Hence the literature spanning 1969 to 1999 shows that although intransal vaccination with inactivated influenza vaccine has been widely investigated, no group has been able to reach the objective of achieving systemic seroconversion equivalent to intramuscular or subcutaneous injection by administering a single dose of vaccine nasally. Furthermore, in order to achieve an effect with multiple administrations of vaccine over time, the doses of antigen used have been considerably greater than the standard conventional dose of 15 μg HA per strain for each vaccinee. The data in fact point towards a need for multiple administrations, and preferably in the presence of strong immunostimulants such as *E. coli* HLT.

Kimura et al. (1988. Acta Paediatr Jpn. 30, 601-3) demonstrated that administration of two doses of inactivated influenza virus by nebuliser was as effective as a single s.c. administration, but that administration of the two doses by intranasal spray was far less effective. Nebulisation generates a very fine spray which reaches the lungs. Thus there

is also an indication in the published clinical trials that a nasal spray may not be effective, and that nebulisation may be better.

The literature further indicates the need for potent adjuvants in intranasal vaccines.

For example, Gluck et al. (cited above) demonstrated that the intranasal administration of influenza vaccine in the absence of adjuvant is significantly less efficient than in the presence of adjuvant. The authors show that even two administrations of vaccine lacking adjuvant are unable to induce the same seroconversion achieved by subcutaneous administration.

Hashigucci et al. 1996 (Vaccine 14, 113-9) demonstrated that two intranasal administrations, four weeks apart, of a split influenza vaccine adjuvanted with a mixture of *E. coli* Heat labile toxin and its B-subunit (LTB) resulted in a seroconversion rate of 50%. In the absence of adjuvant only 31% seroconversion was obtained. Typically s.c. administration results in 75-90% seroconversion.

Thus, the literature clearly indicates that in order to achieve equivalent systemic seroconversion to that obtained with conventional flu vaccines, more than one administration is required, and in addition the vaccine should be adjuvanted with a toxin.

It has now been discovered that non-live influenza virus antigen can be used in a commercially viable intranasal flu vaccine. In particular, a single administration of an intranasal influenza virus vaccine preparation stimulates systemic immunity at a protective level. Furthermore, this meets the international criteria for an effective flu vaccine. More specifically, intranasal administration of a non-live influenza virus antigen preparation can produce a systemic seroconversion (4-fold increase in anti-HA titres) equivalent to that obtained by s.c. administration of the same vaccine. Surprisingly, the influenza antigen can be provided at a significantly lower dose than is indicated in the prior art.

A single nasal administration of a standard dose of inactivated influenza virus resulting in seroconversion equivalent to that obtained by injection has not previously been reported.

The invention provides for the first time a single administration influenza vaccine for intranasal delivery.

Thus, the invention provides in one aspect the use of a non-live influenza virus antigen preparation in the manufacture of a vaccine formulation for a one-dose nasal vaccination against influenza. The vaccine may be administered in a mono-dose format or a bi-dose format (generally one sub-dose for each nostril).

The invention provides in another aspect the use of a low dose of non-live influenza virus antigen material in the manufacture of a mucosal vaccine for immunisation against influenza.

Preferably, the non-live influenza virus antigen preparation contains at least one surfactant which may be in particular a non-ionic surfactant. Preferably the non-ionic surfactant is at least one surfactant selected from the group consisting of the octyl- or nonylphenoxy polyoxyethanols (for example the commercially available Triton ™ series), polyoxyethylene sorbitan esters (Tween ™ series) and polyoxythylene ethers or esters of general formula (I):

$$(I) \quad HO(CH_2CH_2O)_n\text{-}A\text{-}R$$

wherein n is 1-50, A is a bond or –C(O)-, R is $C_{1\text{-}50}$ alkyl or phenyl $C_{1\text{-}50}$ alkyl, and combinations of two or more of these.

Preferred surfactants falling within formula (I) are molecules in which n is 4-24, more preferably 6-12, and most preferably 9; the R component is $C_{1\text{-}50}$, preferably C_4-C_{20} alkyl and most preferably C_{12} alkyl.

Octylphenoxy polyoxyethanols and Polyoxyethylene sorbitan esters are described in "Surfactant systems" Eds: Attwood and Florence (1983, Chapman and Hall). Octylphenoxy polyoxyethanols (the octoxynols), including t-

octylphenoxypolyethoxyethanol (Triton X-100 ™) is also described in Merck Index Entry 6858 (Page 1162, 12th Edition, Merck & Co. Inc., Whitehouse Station, N.J., USA; ISBN 0911910-12-3). The polyoxyethylene sorbitan esters, including polyoxyethylene sorbitan monooleate (Tween 80 ™) are described in Merck Index Entry 7742 (Page 1308, 12th Edition, Merck & Co. Inc., Whitehouse Station, N.J., USA; ISBN 0911910-12-3). Both may be manufactured using methods described therein, or purchased from commercial sources such as Sigma Inc.

Particularly preferred non-ionic surfactants include Triton X-45, t-octylphenoxy polyethoxyethanol (Triton X-100), Triton X-102, Triton X-114, Triton X-165, Triton X-205, Triton X-305, Triton N-57, Triton N-101, Triton N-128, Breij 35, polyoxyethylene-9-lauryl ether (laureth 9) and polyoxyethylene-9-stearyl ether (steareth 9). Triton X-100 and laureth 9 are particularly preferred. Also particularly preferred is the polyoxyethylene sorbitan ester, polyoxyethylene sorbitan monooleate (Tween 80™).

Further suitable polyoxyethylene ethers of general formula (I) are selected from the following group: polyoxyethylene-8-stearyl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether.

Alternative terms or names for polyoxyethylene lauryl ether are disclosed in the CAS registry. The CAS registry number of polyoxyethylene-9 lauryl ether is: 9002-92-0. Polyoxyethylene ethers such as polyoxyethylene lauryl ether are described in the Merck index (12th ed: entry 7717, Merck & Co. Inc., Whitehouse Station, N.J., USA; ISBN 0911910-12-3). Laureth 9 is formed by reacting ethylene oxide with dodecyl alcohol, and has an average of nine ethylene oxide units.

The ratio of the length of the polyoxyethylene section to the length of the alkyl chain in the surfactant (*i.e.* the ratio of n: alkyl chain length), affects the solubility of this class of surfactant in an aqueous medium. Thus, the surfactants of the present invention may be in solution or may form particulate structures such as micelles or vesicles. As a solution, the surfactants of the present invention are safe, easily sterilisable, simple to administer, and may be manufactured in a simple fashion without the GMP and QC

issues associated with the formation of uniform particulate structures. Some polyoxyethylene ethers, such as laureth 9, are capable of forming non-vesicular solutions. However, polyoxyethylene-8 palmitoyl ether ($C_{18}E_8$) is capable of forming vesicles. Accordingly, vesicles of polyoxyethylene-8 palmitoyl ether in combination with at least one additional non-ionic surfactant, can be employed in the formulations of the present invention.

~~Preferably, the polyoxyethylene ether used in the formulations of the present invention~~ has haemolytic activity. The haemolytic activity of a polyoxyethylene ether may be measured *in vitro*, with reference to the following assay, and is as expressed as the highest concentration of the surfactant which fails to cause lysis of the red blood cells:

1. Fresh blood from guinea pigs is washed with phosphate buffered saline (PBS) 3 times in a desk-top centrifuge. After re-suspension to the original volume the blood is further diluted 10 fold in PBS.
2. 50 µl of this blood suspension is added to 800 µl of PBS containing two-fold dilutions of detergent.
3. After 8 hours the haemolysis is assessed visually or by measuring the optical density of the supernatant. The presence of a red supernatant, which absorbs light at 570 nm indicates the presence of haemolysis.
4. The results are expressed as the concentration of the first detergent dilution at which hemolysis no longer occurs.

Within the inherent experimental variability of such a biological assay, the polyoxyethylene ethers, or surfactants of general formula (I), of the present invention preferably have a haemolytic activity, of approximately between 0.5-0.0001%, more preferably between 0.05-0.0001%, even more preferably between 0.005-0.0001%, and most preferably between 0.003-0.0004%. Ideally, said polyoxyethylene ethers or esters should have a haemolytic activity similar (*i.e.* within a ten-fold difference) to that of either polyoxyethylene-9 lauryl ether or polyoxyethylene-8 stearyl ether.

Two or more non-ionic surfactants from the different groups of surfactants described may be present in the vaccine formulation described herein. In particular, a

combination of a polyoxyethylene sorbitan ester such as polyoxyethylene sorbitan monooleate (Tween 80™) and an octoxynol such as t-octylphenoxypolyethoxyethanol (Triton) X-100™ is preferred. Another particularly preferred combination of non-ionic surfactants comprises laureth 9 plus a polyoxyethylene sorbitan ester or an octoxynol or both.

Preferably the or each non-ionic surfactant is present in the final vaccine formulation at ~~a concentration of between 0.001 to 20%, more preferably 0.01 to 10%, and most~~ preferably up to about 2% (w/v). Where one or two surfactants are present, these are generally present in the final formulation at a concentration of up to about 2% each, typically at a concentration of up to about 0.6% each. One or more additional surfactants may be present, generally up to a concentration of about 1% each and typically in traces up to about 0.2% or 0.1 % each. Any mixture of surfactants may be present in the vaccine formulations according to the invention.

Non-ionic surfactants such as those discussed above have preferred concentrations in the final vaccine composition as follows: polyoxyethylene sorbitan esters such as Tween 80™: 0.01 to 1%, most preferably about 0.1% (w/v); octyl- or nonylphenoxy polyoxyethanols such as Triton X-100™ or other detergents in the Triton series: 0.001 to 0.1%, most preferably 0.005 to 0.02 % (w/v); polyoxyethylene ethers of general formula (I) such as laureth 9: 0.1 to 20 %, preferably 0.1 to 10 % and most preferably 0.1 to 1 % or about 0.5% (w/v).

The non-live flu antigen preparation may be selected from the group consisting of split virus antigen preparations, subunit antigens (either recombinantly expressed or prepared from whole virus), inactivated whole virus which may be chemically inactivated with e.g. formaldehyde, β-propiolactone or otherwise inactivated e.g. U.V. or heat inactivated. Preferably the antigen preparation is either a split virus preparation, or a subunit antigen prepared from whole virus, particularly by a splitting process followed by purification of the surface antigen.

In a preferred embodiment, the vaccine formulation comprises a split flu virus preparation in combination with one or more non-ionic surfactants. The one or more non-ionic surfactants may be residual from the process by which the split flu antigen preparation is produced, and/or added to the antigen preparation later. It is believed that the split flu antigen material may be stabilised in the presence of a non-ionic surfactant, though it will be understood that the invention does not depend upon this necessarily being the case.

The invention provides in another aspect the use of a non-live influenza virus antigen preparation, preferably a split flu virus preparation, in the manufacture of a one-dose intranasal influenza vaccine without an added immunostimulant. In the context of this invention, an immunostimulant is a substance which is capable of directly stimulating cells of the immune system, as opposed to only indirectly stimulating e.g. by acting as a carrier for an antigen that itself has a stimulatory effect when in combination with the carrier.

In an alternative embodiment of the present invention, the formulation further comprises adjuvants or immunostimulants including Cholera toxin and its B subunit, detoxified lipid A from any source, Monophosphoryl Lipid A and its non-toxic derivative 3-de-O-acylated monophosphoryl lipid A (e.g. as described in UK patent no. GB 2,220,211), saponins such as Quil A (derived from the bark of the South American tree *Quillaja Saponaria Molina*), and fractions thereof, including QS21 and QS17 (US 5,057,540; Kensil, C. R., *Crit Rev Ther Drug Carrier Syst*, 1996, 12 (1-2):1-55; EP 0 362 279 B1; Kensil *et al.* (1991. J. Immunology vol 146, 431-437; WO 99/10008) and the oligonucleotide adjuvant system containing an unmethylated CpG dinucleotide (as described in WO 96/02555).

In a further aspect, the invention provides a pharmaceutical kit comprising an intranasal spray device and a one-dose non-live influenza virus vaccine. Preferably the device is a bi-dose delivery device for two sub-doses of vaccine.

The low dose of haemagglutinin according to the invention is preferably a haemagglutinin dose comparable to the dose in the current commercial flu vaccines.

Thus the preferred low dose is preferably not more than about 30 μg, more preferably not more than about 15 μg of haemagglutinin per influenza strain. This equates to normally somewhere between 0.1 and 2 μg /kg bodyweight. Preferably but not necessarily the low dose vaccines of the invention are administered as a one-dose vaccine e.g. in two sub-doses.

Advantageously, a vaccine dose according to the invention is provided in a smaller volume than the conventional injected split flu vaccines, which are generally 0.5 or 1 ml per dose. The low volume doses according to the invention are preferably below 500 μl, more preferably below 300 μl and most preferably not more than about 200 μl or less per dose. When two sub-doses are given, the preferred volume per sub-dose is half of the total dose volumes mentioned above.

Thus, a preferred vaccine dose according to the invention is a dose with a low antigen dose in a low volume, e.g. about 15 μg or about 7.5 μg HA (per strain) in a volume of 200 μl.

The invention also provides a method for the prophylaxis of influenza infection or disease in a subject which method comprises administering to the subject a one-dose non-live influenza vaccine via a mucosal surface.

The invention further provides a method for prophylaxis of influenza infection or disease in a subject which method comprises administering to the subject a low dose of a non-live influenza virus vaccine via a mucosal surface.

Preferably the vaccine is administered intranasally.

Most preferably, the vaccine is administered locally to the nasopharyngeal area, preferably without being inhaled into the lungs. It is desirable to use an intranasal delivery device which delivers the vaccine formulation to the nasopharyngeal area, without or substantially without it entering the lungs.

Preferred devices for intranasal administration of the vaccines according to the invention are spray devices. Suitable commercially available nasal spray devices include Accuspray™ (Becton Dickinson). Nebulisers produce a very fine spray which can be easily inhaled into the lungs and therefore does not efficiently reach the nasal mucosa. Nebulisers are therefore not preferred.

Preferred spray devices for intranasal use do not depend for their performance on the pressure applied by the user. Pressure threshold devices are particularly useful since liquid is released from the nozzle only when a threshold pressure is attained. These devices make it easier to achieve a spray with a regular droplet size. Pressure threshold devices suitable for use with the present invention are known in the art and are described for example in WO 91/13281 and EP 311 863 B. Such devices are commercially available from Pfeiffer GmbH and are also described in Bommer, R. Pharmaceutical Technology Europe, Sept 1999.

Preferred intranasal devices produce droplets (measured using water as the liquid) in the range 1 to 200μm, preferably 10 to 120μm. Below 10μm there is a risk of inhalation, therefore it is desirable to have no more than about 5% of droplets below 10μm. Droplets above 120μm do not spread as well as smaller droplets, so it is desirable to have no more than about 5% of droplets exceeding 120μm.

Bi-dose delivery is a further preferred feature of an intranasal delivery system for use with the vaccines according to the invention. Bi-dose devices contain two subdoses of a single vaccine dose, one sub-dose for each nostril. Alternatively, a monodose device may be used for administering the vaccines according to the invention.

The invention provides in a further aspect a pharmaceutical kit comprising an intranasal administration device as described herein containing a vaccine formulation according to the invention.

The invention is not necessarily limited to spray delivery of liquid formulations. Vaccines according to the invention may be administered in other forms eg as a powder.

The non-live influenza virus preparations may be derived from the conventional embryonated egg method, or they may be derived from any of the new generation methods using tissue culture to grow the virus. Suitable cell substrates for growing the virus include for example dog kidney cells such as MDCK or cells from a clone of MDCK, MDCK-like cells, monkey kidney cells such as AGMK cells including Vero cells, or any other cell type suitable for the production of influenza virus for vaccine purposes. Suitable cell substrates include human cells e.g. MRC-5 cells. Suitable cell substrates are not limited to cell lines; for example primary cells such as chicken embryo fibroblasts are also included.

The influenza virus antigen preparation may be produced by any of a number of commercially applicable processes, for example the split flu process described in patent no. DD 300 833. Traditionally split flu was produced using a solvent/detergent treatment, such as ether in combination with Tween™ and this process is still used in some production facilities. Other splitting agents employed include detergents or proteolytic enzymes or bile salts, for example sodium deoxycholate as described in patent no. DD 155 875. Detergents that can be used as splitting agents include cationic detergents e.g. cetyl trimethyl ammonium bromide (CTAB), other ionic detergents e.g. lauryl sulfate, taurodeoxycholate , or non-ionic detergents such as the ones described above, or combinations of any two or more detergents.

Further suitable splitting agents which can be used to produce split flu virus preparations include:

1. Bile acids and derivatives thereof including: cholic acid, deoxycholic acid, chenodeoxy colic acid, lithocholic acid ursodeoxycholic acid, hyodeoxycholic acid and derivatives like glyco-, tauro-, amidopropyl-1-propanesulfonic-, amidopropyl-2-hydroxy-1-propanesulfonic derivatives of the aforementioned bile acids, or N,N-bis (3DGluconoamidopropyl) deoxycholamide. A particular example is sodium

deoxycholate (NaDOC) which may be present in trace amounts in the final vaccine dose.

2. Alkylglycosides or alkylthioglycosides, where the alkyl chain is between C6 - C18 typical between C8 and C14, sugar moiety is any pentose or hexose or combinations thereof with different linkages, like 1-> 6, 1->5, 1->4, 1->3, 1-2. The alkyl chain can be saturated unsaturated and/or branched.

3. Derivatives of 2 above, where one or more hydroxyl groups, preferably the 6 hydroxyl group is/are modified, like esters, ethoxylates, sulphates, ethers, carbonates, sulphosuccinates, isethionates, ethercarboxylates, quarternary ammonium compounds.

4. Acyl sugars, where the acyl chain is between C6 and C18, typical between C8 and C12, sugar moiety is any pentose or hexose or combinations thereof with different linkages, like 1-> 6, 1->5, 1->4, 1->3, 1-2. The acyl chain can be saturated or unsaturated and/or branched, cyclic or non-cyclic, with or without one or more heteroatoms e.g. N, S, P or O.

5. Sulphobetaines of the structure R-N,N-(R1,R2)-3-amino-1-propanesulfonate, where R is any alkyl chain or arylalkyl chain between C6 and C18, typical between C8 and C16. The alkyl chain R can be saturated, unsaturated and/or branched. R1 and R2 are preferably alkyl chains between C1 and C4, typically C1, or R1, R2 can form a heterocyclic ring together with the nitrogen.

6. Betains of the structure R-N,N-(R1,R2)-glycine, where R is any alkyl chain between C6 and C18, typical between C8 and C16. The alkyl chain can be saturated unsaturated and/or branched. R1 and R2 are preferably alkyl chains between C1 and C4, typically C1, or R1 and R2 can form a heterocyclic ring together with the nitrogen.

7. N,N-dialkyl-glucamides, of the Structure R-(N-R1)-glucamide, where R is any alkylchain between C6 and C18, typical between C8 and C12. The alkyl chain can be saturated unsaturated and/or branched or cyclic. R1 and R2 are alkyl chains between

C1 and C6, typically C1. The sugar moiety might be modified with pentoses or hexoses.

8. Quarternary ammonium compounds of the structure R, $-N^+$ (-R1, -R2, -R3), where R is any alkylchain between C6 and C20, typically C20. The alkyl chain can be saturated unsaturated and/or branched. R1, R2 and R3 are preferably alkyl chains between C1 and C4, typically C1, or R1, R2 can form a heterocyclic ring together with the nitrogen. A particular example is cetyl trimethyl ammonium bromide (CTAB).

The preparation process will include a number of different filtration and/or other separation steps such as ultracentrifugation, ultrafiltration, zonal centrifugation and chromatography (e.g. ion exchange) steps in a variety of combinations, and optionally an inactivation step eg with formaldehyde or β-propiolactone or U.V. which may be carried out before or after splitting. The splitting process may be carried out as a batch, continuous or semi-continuous process.

Preferred split flu vaccine antigen preparations according to the invention comprise a residual amount of Tween 80 and/or Triton X-100 remaining from the production process. Preferably both Tween 80 and Triton X-100 are present. The preferred ranges for the final concentrations of these non-ionic surfactants in the vaccine dose are:

Tween 80: 0.01 to 1%, more preferably about 0.1% (v/v)

Triton X-100: 0.001 to 0.1 (% w/v), more preferably 0.005 to 0.02% (w/v).

The presence of the combination of these two surfactants, in low concentrations, was found to promote the stability of the antigen in solution. It is possible that this enhanced stability rendered the antigen more immunogenic nasally than previous formulations have been. Such an enhancement could arise from a prevalence of small antigen aggregates or the enhancement of the native conformation of the antigen. It will be appreciated that the invention does not depend upon this theoretical explanation being correct.

In a particular embodiment, the preferred split virus preparation also contains laureth 9, preferably in the range 0.1 to 20%, more preferably 0.1 to 10% and most preferably 0.1 to 1% (w/v).

The vaccines according to the invention generally contain not more than 25% (w/v) of detergent or surfactant, preferably less than 15% and most preferably not more than about 2%.

The invention will now be further described in the following, non-limiting examples.

EXAMPLES

Example 1 – Preparation of split influenza vaccine

Monovalent split vaccine was prepared according to the following procedure.

Preparation of virus inoculum

On the day of inoculation of embryonated eggs a fresh inoculum is prepared by mixing the working seed lot with a phosphate buffered saline containing gentamycin sulphate at 0.5 mg/ml and hydrocortisone at 25 µg/ml. (virus strain-dependent). The virus inoculum is kept at 2-8°C.

Inoculation of embryonated eggs

Nine to eleven day old embryonated eggs are used for virus replication. Shells are decontaminated. The eggs are inoculated with 0.2 ml of the virus inoculum. The inoculated eggs are incubated at the appropriate temperature (virus strain-dependent) for 48 to 96 hours. At the end of the incubation period, the embryos are killed by cooling and the eggs are stored for 12-60 hours at 2-8°C.

Harvest

The allantoic fluid from the chilled embryonated eggs is harvested. Usually, 8 to 10 ml of crude allantoic fluid is collected per egg. To the crude monovalent virus bulk 0.100 mg/ml thiomersal is optionally added.

Concentration and purification of whole virus from allantoic fluid

1. Clarification

The harvested allantoic fluid is clarified by moderate speed centrifugation (range: 4000 – 14000 g).

2. Adsorption step

To obtain a $CaHPO_4$ gel in the clarified virus pool, 0.5 mol/L Na_2HPO_4 and 0.5mol/L $CaCl_2$ solutions are added to reach a final concentration of $CaHPO_4$ of 1.5 g to 3.5 g $CaHPO_4$/litre depending on the virus strain.

After sedimentation for at last 8 hours, the supernatant is removed and the sediment containing the influenza virus is resolubilised by addition of a 0.26 mol/L EDTA-Na_2 solution, dependent on the amount of $CaHPO_4$ used.

3. Filtration

The resuspended sediment is filtered on a 6µm filter membrane.

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4. Sucrose gradient centrifugation

The influenza virus is concentrated by isopycnic centrifugation in a linear sucrose gradient (0.55 % (w/v)) containing 100 µg/ml Thiomersal. The flow rate is 8 – 15 litres/hour.

At the end of the centrifugation, the content of the rotor is recovered by four different fractions (the sucrose is measured in a refractometer):

- fraction 1 55-52% sucrose
- fraction 2 approximately 52-38% sucrose
- fraction 3 38-20% sucrose*
- fraction 4 20- 0% sucrose

* virus strain-dependent: fraction 3 can be reduced to 15% sucrose.

For further vaccine preparation, only fractions 2 and 3 are used.

Fraction 3 is washed by diafiltration with phosphate buffer in order to reduce the sucrose content to approximately below 6%. The influenza virus present in this diluted fraction is pelleted to remove soluble contaminants.

The pellet is resuspended and thoroughly mixed to obtain a homogeneous suspension. Fraction 2 and the resuspended pellet of fraction 3 are pooled and phosphate buffer is added to obtain a volume of approximately 40 litres. This product is the monovalent whole virus concentrate.

5. Sucrose gradient centrifugation with sodium deoxycholate

The monovalent whole influenza virus concentrate is applied to a ENI-Mark II ultracentrifuge. The K3 rotor contains a linear sucrose gradient (0.55 % (w/v)) where a sodium deoxycholate gradient is additionally overlayed. Tween 80 is present during splitting up to 0.1 % (w/v). The maximal sodium deoxycholate concentration is 0.7-1.5 % (w/v) and is strain dependent. The flow rate is 8 – 15 litres/hour.

~~At the end of the centrifugation, the content of the rotor is recovered by three different~~ fractions (the sucrose is measured in a refractometer) Fraction 2 is used for further processing. Sucrose content for fraction limits (47-18%) varies according to strains and is fixed after evaluation:

6. Sterile filtration

The split virus fraction is filtered on filter membranes ending with a 0.2 µm membrane. Phosphate buffer containing 0.025 % (w/v) Tween 80 is used for dilution. The final volume of the filtered fraction 2 is 5 times the original fraction volume.

7. Inactivation

The filtered monovalent material is incubated at 22 ± 2°C for at most 84 hours (dependent on the virus strains, this incubation can be shortened). Phosphate buffer containing 0.025% Tween 80 is then added in order to reduce the total protein content down to max. 250 µg/ml. Formaldehyde is added to a final concentration of 50 µg/ml and the inactivation takes place at 20°C ± 2°C for at least 72 hours.

8. Ultrafiltration

The inactivated split virus material is concentrated at least 2 fold in a ultrafiltration unit, equipped with cellulose acetate membranes with 20 kDa MWCO. The Material is subsequently washed with phosphate buffer containing 0.025 % (w/v) Tween 80 and following with phosphate buffered saline containing 0.01 % (w/v) Tween.

9. Final sterile filtration

The material after ultrafiltration is filtered on filter membranes ending with a 0.2 µm membrane. The final concentration of Haemagglutinin, measured by SRD (method recommended by WHO) should exceed 450 µg/ml.

10. Storage

The monovalent final bulk is stored at 2 – 8°C for a maximum of 18 months.

Purity

Purity was determined by O.D. scanning of Coomassie-stained polyacrylamide gels. Peaks were determined manually. Sample results are given in Table 1:

Table 1

Viral Proteins (HA, NP, M) %					Other viral and host-cell derived proteins %
H3N2	**HA dimer**	**HA1 + 2**	**NP**	**M**	
A/Syd/5/97	10.34	22.34	25.16	37.33	4.83
A/Nan933/95	8.17	15.8	40.09	30.62	5.32
B					
B/Har/7/94	5.71[2]	24.07	15.64	50	4.58
B/Yam/166/98	0.68	27.62	21.48	46.02	4.2
H1N1					
A/Tex/36/91		33.42	24.46	34.33	7.79
A/Bei/262/95		32.73	35.72	27.06	4.49
H2N2					
A/sing/1/57	2.8	39.7	21.78	32.12	3.6

[1] = 100 % minus all non-identified peaks

Example 2 – Preparation of vaccine doses from bulk vaccine

Final vaccine is prepared by formulating a trivalent vaccine from the monovalent bulk with the detergent concentrations adjusted as required.

Water for injection, PBS pH 7.4 10x concentrated, Tween 80 and Triton X-100 are mixed to obtain the required final concentrations (PBS 1x concentrated, Tween 80 0.15% and Triton X-100 0.02%) . The three following inactivated split virions are added with 10 minutes stirring in between:

30µg HA A/Beijing/262/95 (H1N1)
30µg HA A/Sydney/5/97 (H3N2)
30µg HA B/Harbin/7/94

After 15minutes stirring pH is adjusted to 7.2+/-0.2.
The dose volume is 200µl.
In the formulation adjuvanted with laureth 9, the laureth 9 is added prior to pH adjustment to obtain a final concentration of 0.5% (w/v).

Example 3 – Methods used to measure antibody responses in sera

Detection of specific anti-Flu and total IgA in human nasal secretions by ELISA

Collection method for human nasal secretions

Two wicks are applied against the inferior turbinate (one in each nostril) of the volunteer. Wicks are left in the nose for 1 minute before being placed in 2 ml of NaCl 0.9%, BSA 1% and sodium azide 0.1% (preservative buffer). All the samples are left for a 2 hours period on ice. The wicks are then pressed to recover the antibodies. Following centrifugation (10', 2000g, 4°C) the fluids of all samples are collected, aliquoted and frozen at –20°C until the date of test. The pellets are suspended in 400µl of physiological water and microscopically observed for blood cells contamination.

After collection using nasal wicks and treatment of human nasal secretions, the detection of total and specific anti-FLU IgA is realized with two different ELISAs:

Capture ELISA for detection of total IgA

Total IgA are captured with anti-human IgA polyclonal affinity purified Ig immobilized on microtiter plates and subsequently detected using a different polyclonal anti-human IgA affinity purified Ig coupled to peroxidase.

A purified human sIgA is used as a standard to allow the quantification of sIgA in the collected nasal secretions.

3 references of purified human sIgA are used as low, medium and high references in this assay.

Direct ELISA for detection of specific anti-FLU IgA

Three different ELISAs are performed, one on each FLU strain present in the vaccine formulation.

Specific anti-FLU IgA are captured with split inactivated FLU antigens coated on microtiter plates and subsequently detected using the same different polyclonal anti-human IgA affinity purified Ig coupled to peroxidase as the one used for the total IgA ELISA.

Reagents

Biological reagents

- Goat anti-Human IgA affinity purified Ig.(Sigma I-0884)
- Purified Human secretory IgA (ICN-Cappel 55905) (Standard for total IgA quantification)
- Human secretory IgA (Colostrum) (Biogenesis 5111-5504) (reference Bio for total IgA), diluted to obtain low, medium and high references

- Purified Human IgA (Sigma I-1010) (reference Sig for total IgA)
- Negative reference for specific anti-FLU ELISA (pool of nasal secretions with undetectable responses against the 3 strains; cut-off = 0.6 OD_{450nm})
- Positive low reference for specific anti- FLU ELISA (pool of nasal secretions with low detectable responses against the 3 strains)
- Positive medium reference for specific anti- FLU ELISA (pool of nasal secretions with medium detectable responses against the 3 strains; cut-off = 0.6 OD)
- Goat anti-Human IgA serum affinity purified HRP conjugated (ICN 674221)
- Split inactivated egg derived antigen A/Beijing/262/95 H1N1
- Split inactivated egg derived antigen A/Sydney/5/97 H3N2
- Split inactivated egg derived antigen B/Harbin/7/94

Reagents preparation

- Saturation buffer (PBS, Tween 20 0.1%, BSA 1%, NCS 4%)
- NaCl T20 (NaCl 9g/l, Tween 20 0.05%)

Method

Total human IgA detection

- Add 100 µl/well of goat polyclonal anti human IgA at 1 µg/ml in DPBS and incubate overnight at 4°C.
- Add 200 µl/well of saturation buffer and incubate for 1 hour at 37°C.
- Add in the first row: 100 µl/well of two-fold dilutions of the standard IgA in saturation buffer starting from 250 ng/ml down to 0.12 ng/ml.
- Add in the other rows: 100 µl/well of two-fold dilutions of the samples (nasal fluids) in saturation buffer starting from 1/100 down to 1/102400, add 100µl of saturation buffer in the column 12 and incubate for 2 hours at 22°C.

- Wash the plates four times in NaCl T20.
- Add 100 µl/well of goat peroxidase-conjugated anti human IgA diluted in saturation buffer at 1/10000 and incubate for 1 1/2 hour at 22°C.
- Wash the plates four times in NaCl T20.
- Add 100 µl/well of TMB (tetramethylbenzidine) and incubate at room temperature for 10 min. in the dark.
- Stop the reaction by adding 100µl/well of H_2SO_4 0.4N.
- Measure the absorbance (OD) of each plate using a spectrophotometer at 450 nm with a reference at 630 nm.

Specific anti-FLU IgA detection

- Add 100 µl/well of each strain of FLU virus at 1 µg/ml in DPBS and incubate overnight at 4°C.
- Add 200 µl/well of saturation buffer and incubate for 1 hour at 37°C.
- Add 100 µl/well of two-fold dilutions of the samples in saturation buffer starting from 1/5 down to 1/640 and incubate the plates for 2 hours at 22°C.
- Wash the plates four times in NaCl T20.
- Add 100 µl/well of goat peroxidase-conjugated anti human IgA diluted in saturation buffer at 1/10000 and incubate for 1 1/2 hour at 22°C.
- Wash the plates four times in NaCl T20.
- Add 100 µl/well of TMB (tetramethylbenzidine) and incubate at room temperature for 10 min. in the dark.
- Stop the reaction by adding 100µl/well of H_2SO_4 0.4N.
- Measure the absorbance (OD) of each plate using a spectrophotometer at 450 nm with a reference at 630 nm.

Results - expression and calculations

Total IgA expression

The results are expressed as µg of total IgA in 1 ml of nasal fluids, using a Softmaxpro program.

Specific anti-Flu IgA expression

The results are expressed as end-point unit titer, which are calculated as the inverse of the last dilution which gives an OD_{450nm} above the cut off (OD_{450nm} = 0.6).

The cut off value is defined as the highest optical density of the negative reference (see validation protocol) at a dilution of 1/5. The limit of detection corresponding to the end-point unit titer at the cut off can thus be calculated as being 5 end-point units. Samples with a titer ≤ 5 end-point unit will be considered as negative and samples with a titer > 5 end-point unit will be considered as positive.

The final results of a sample are expressed as follows:

Normalization of the specific response by calculating the ratio between the specific response and the total IgA concentration: end-point unit/µg total IgA (most commonly used calculation method in the literature).

Hemagglutination Inhibition (HAI) activity of Flu-specific serum Abs

Sera (50 µl) are treated with 200 µl RDE (receptor destroying enzyme) for 16 hours at 37°C. The reaction is stopped with 150 µl 2.5% Na citrate and the sera are inactivated at 56°C for 30 min. A dilution 1:10 is prepared by adding 100 µl PBS. Then, a 2-fold dilution series is prepared in 96 well plates (V-bottom) by diluting 25 µl serum (1:10) with 25 µl PBS. 25 µl of the reference antigens are added to each well at a concentration of 4 hemagglutinating units per 25 µl. Antigen and antiserum dilution are mixed using a microtiter plate shaker and incubated for 60 minutes at room temperature. 50 µl chicken red blood cells (RBC) (0.5%) are then added and the RBCs are allowed to sediment for 1 hour at RT. The HAI titre corresponds to the inverse of the last serum dilution that completely inhibits the virus-induced hemagglutination.

Example 4 – A comparison of the immunogenicity of an intranasal split influenza vaccine compared to a licensed conventional parenteral vaccine (Fluarix™) in healthy adult subjects.

Formulations used in the study

Two formulations (A,B) of egg-derived split influenza antigens were evaluated. A is an intranasal formulation and B is the Fluarix™/α-Rix® given intramuscularly.
The formulations contain three inactivated split virion antigens prepared from the WHO recommended strains of the 1998/1999 season.

The device used for administration of the vaccines was the Accuspray™ intranasal syringe from Becton Dickinson. The device works on a similar basis to a conventional syringe, but has a special tip containing spiral channels which result in the production of a spray when even pressure is exerted on the plunger. 100µl of the A formulation was sprayed in each nostril.

Composition of the formulations.

The intranasal formulation (A) contained the following inactivated split virions:

1. 30µg HA A/beijing/262/95 (H1N1)
2. 30µg HA A/Sydney/5/97 (H3N2)
3. 30µg HA of B/Harbin/7/94

and phosphate buffered saline pH 7.4± 0.1, Tween 80 0.1%, Triton X-100 0.015%, Na deoxycholate 0.0045% and thiomersal below 35µg/ml.

The volume of one dose was 200µl (100µl sub-doses for each nostril).

The comparator Fluarix™/α-Rix® is the SmithKline Beecham Biologicals' commercial inactivated trivalent split influenza vaccine. The dose of 500µl was administered intramuscularly.

This dose contains:

15μg HA of the three strains mentioned above, Tween 80 between 500 and 1000 μg per ml (0.05%-0.1%), Triton X-100 between 50 and 170μg/ml (0.005%-0.017%), sodium deoxycholate maximum 100μg/ml, thiomersal 100μg/ml and phosphate buffered saline pH between 6.8 and 7.5.

Immunogenicity Study

An open, controlled and randomised study evaluated the immunogenicity of an intranasal split influenza vaccine formulated with Tween 80 & Triton X-100 compared to the conventional parenteral vaccine (i.e. Fluarix™). Twenty healthy adult subjects (aged 18-40 years) received one dose of Fluarix™ and ten subjects received one dose of the intranasal influenza vaccine. The intranasal formulation (200μl) contained the following inactivated virions: 30μg of haemagglutinin A/Beijing/262/95 (H1N1), 30μg of haemagglutinin A/Sydney/5/97 (H3N2), 30μg of haemagglutinin B/Harbin/7/94 with phosphate buffered saline (pH 7.4 ± 0.1), Tween 80 (0.1%), Triton X-100 (0.015%), sodium deoxycholate (0.0045%) and thiomersal (<35μg/ml).

There was an eight-day follow-up period for solicited local and general symptoms and both vaccines were well-tolerated regarding safety and reactogenicity. No serious adverse events related to vaccination were reported.

The immunogenicity of the vaccines was examined by assessing the serum haemagglutination inhibition (HI) titres to determine the seroconversion rate (defined as the percentage of vaccinees who have at least a 4-fold increase in serum HI titres on day 21 compared to day 0, for each vaccine strain), conversion factor (defined as the fold increase in serum HI Geometric Mean Titres (GMTs) on day 21 compared to day 0, for each vaccine strain) and seroprotection rate (defined as the percentage of vaccinees with a serum HI titre ≥40 after vaccination (for each vaccine strain) that is accepted as indicating protection). In addition, the mucosal IgA antibody response was assessed by Enzyme Linked Immunosorbent Assay (ELISA).

HI seropositivity, serconversion and seroprotection rates twenty-one days after one dose of Fluarix™ or the intranasal formulation can be seen in Table 2.

Table 2:

HI seropositivity, serconversion and seroprotection rates at 21 days post dose 1

Strain	Group	*Timing*	N	Seropositivity		Seroprotection		Seroconversion	
				n	%	n	%	n	%
A/Beijing	Intranasal vaccine plus Tween 80 & Titron X100	Day 0	20	4	20.0	0	0.0		
		Day 21	20	17	85.0	15	75.0	15	75.0
	Fluarix™	Day 0	19	4	21.1	3	15.8		
		Day 21	19	19	100.0	18	94.7	19	100.0
A/Sydney	Intranasal vaccine plus Tween 80 & Titron X100	Day 0	20	13	65.0	3	15.0		
		Day 21	20	20	100.0	19	95.0	15	75.0
	Fluarix™	Day 0	19	14	73.7	1	5.3		
		Day 21	19	19	100.0	18	94.7	16	84.2
B/Harbin	Intranasal vaccine plus Tween 80 & Titron X100	Day 0	20	10	50.0	7	35.0		
		Day 21	20	20	100.0	18	90.0	14	70.0
	Fluarix™	Day 0	19	17	89.5	11	57.9		
		Day 21	19	19	100.0	19	100.0	15	78.9

Seropositivity (n,%) : number and percentage of subjects with titer ≥ 10

Seroprotection (n,%) : number and percentage of subjects with titer ≥ 40

Seroconversion (n,%) : number and percentage of subjects with at least a 4-fold increase in titres from day 0 to day 21

The percentage of subjects with a two-fold or a four-fold increase in the specific/total mucosal IgA antibody ratio between day 21 and day 0 (1 dose) can be seen in Table 3.

Table 3:

Percentages of subjects with a two-fold or a four-fold increase in the specific/total IgA ratio between day 21 and day 0 (1 dose).

Strain	Group	N	2 fold increase (%)	4 fold increase (%)
A/Beijing	Tween & Triton	20	55.0	30.0
	Fluarix™	19	52.6	26.3
A/Sydney	Tween & Triton	20	65.0	45.0
	Fluarix™	19	47.4	5.3
B/Harbin	Tween & Triton	20	40.0	30.0
	Fluarix™	19	26.3	5.3

Summary

The immunogenicity results tabulated above show that the intranasal formulation produced similar levels of seropositivity, seroconversion and seroprotection to those produced by the conventional parenteral vaccine (Fluarix™) twenty-one days after one dose. The intranasal formulation produced a better mucosal IgA response after one dose than the conventional parenteral vaccine (Fluarix™).

Example 5 - A comparison of the immunogenicity of an intranasal split influenza vaccine formulated with laureth 9 and Triton X-100, with the immunogenicity of a licensed conventional parenteral vaccine (Fluarix™) in healthy adult subjects.

An intranasal formulation of egg-derived split influenza antigens, formulated with laureth 9 and Triton X-100 (A) was evaluated and compared with Fluarix™/α-Rix® (B). The formulations contained three inactivated split virion antigens prepared from the WHO recommended strains of the 1998/1999 season. The device used for administration of the vaccines was the Accuspray™ intranasal syringe from Becton Dickinson. The device works on a similar basis to a conventional syringe, but has a

special tip containing spiral channels which result in the production of a spray when even pressure is exerted on the plunger. 100µl of the formulation was sprayed in each nostril.

Composition of the formulation

The intranasal formulation (A) contained the following inactivated split virions:

1. 30µg HA A/beijing/262/95 (H1N1)
2. 30µg HA A/Sydney/5/97 (H3N2)
3. 30µg HA of B/Harbin/7/94

and phosphate buffered saline pH 7.4± 0.1, Tween 80 0.1%, Triton X-100 0.015% , sodium deoxycholate 0.0045% and thiomersal below 35µg/ml.

The volume of one dose was 200µl (100µl sub-doses for each nostril).

Formulation A was adjuvanted with laureth 9 to obtain a final concentration of 0.5% (w/v).

The comparator Fluarix™/α-Rix® (B) is SmithKlineBeecham Biologicals' commercial inactivated trivalent split influenza vaccine, which is administered intramuscularly in a dose of 500µl.

Immunogenicity Study

An open, controlled and randomised study evaluated the immunogenicity of an intranasal split influenza vaccine formulated with laureth 9 supplemented with Tween 80 and Triton X-100 compared to the conventional parenteral vaccine (i.e. Fluarix™). Twenty healthy adult subjects (aged 18-40 years) received one dose of Fluarix™ and ten subjects received one dose (two sub-doses, one per nostril) of the intranasal influenza vaccine.

There was an eight-day follow-up period for solicited local and general symptoms and both vaccines were well-tolerated in relation to safety and reactogenicity. No serious adverse events related to vaccination were reported.

The immunogenicity of the vaccines was examined by assessing the serum haemagglutination inhibition (HI) titres to determine seroconversion rate (defined as the percentage of vaccinees who have at least a 4-fold increase in serum HI titres on day 21 compared to day 0, for each vaccine strain), conversion factor (defined as the fold increase in serum HI Geometric Mean Titres (GMTs) on day 21 compared to day 0, for each vaccine strain) and seroprotection rate (defined as the percentage of vaccinees with a serum HI titre ≥40 after vaccination (for each vaccine strain) that is accepted as indicating protection). In addition, the mucosal IgA antibody response was assessed by Enzyme Linked Immunosorbent Assay (ELISA).

HI seropositivity, serconversion and seroprotection rates twenty-one days after one dose of Fluarix™ or the intranasal formulation can be seen in Table 4.

Table 4: HI seropositivity, serconversion and seroprotection rates at 21 days post dose 1:

Strain	Group	*Timing*	N	Seropositivity		Seroprotection		Seroconversion	
				n	%	n	%	n	%
A/Beijing	Intranasal vaccine plus	Day 0	20	5	25.0	1	5.0		
	Laureth 9	Day 21	20	19	95.0	10	50.0	15	75.0
	Fluarix™	Day 0	19	4	21.1	3	15.8		
		Day 21	19	19	100.0	18	94.7	19	100.0
A/Sydney	Intranasal vaccine plus	Day 0	20	16	80.0	4	20.0		
	Laureth-9	Day 21	20	20	100.0	19	95.0	15	75.0
	Fluarix™	Day 0	19	14	73.7	1	5.3		
		Day 21	19	19	100.0	18	94.7	16	84.2
B/Harbin	Intranasal vaccine plus	Day 0	20	18	90.0	11	55.0		
	Laureth-9	Day 21	20	20	100.0	19	95.0	12	60.0
	Fluarix™	Day 0	19	17	89.5	11	57.9		
		Day 21	19	19	100.0	19	100.0	15	78.9

Seropositivity (n,%) : number and percentage of subjects with titer ≥ 10

Seroprotection (n,%) : number and percentage of subjects with titer ≥ 40

Seroconversion (n,%) : number and percentage of subjects with at least a 4-fold increase in titres from day 0 to day 21

The percentage of subjects with a two-fold or a four-fold increase in the specific/total mucosal IgA antibody ratio between day 21 and day 0 (1 dose) can be seen in Table 5.

Table 5: Percentages of subjects with a two-fold or a four-fold increase in the specific/total IgA ratio between day 21 and day 0 (1 dose).

Strain	Group	N	2 fold increase (%)	4 fold increase (%)
A/Beijing	Laureth-9	20	50.0	20.0
	Fluarix™	19	52.6	26.3
A/Sydney	Laureth-9	20	55.0	25.0
	Fluarix™	19	47.4	5.3
B/Harbin	Laureth-9	20	15.0	10.0
	Fluarix™	19	26.3	5.3

Summary

The immunogenicity results tabulated above show that the intranasal formulation produced similar levels of seropositivity, seroconversion and seroprotection to the conventional parenteral vaccine (Fluarix™) twenty-one days after one dose. The intranasal formulation generally produced a better mucosal IgA response after one dose than the conventional parenteral vaccine (Fluarix™).

CLAIMS

1. The use of a non-live influenza virus antigen preparation in the manufacture of a vaccine formulation for a one-dose intranasal vaccination against influenza.

2. The use according to claim 1 wherein the influenza virus antigen preparation is selected from the group consisting of split virus antigen preparations, subunit antigens, chemically or otherwise inactivated whole virus.

3. The use according to claim 2 wherein the formulation comprises at least one surfactant.

4. The use according to claim 2 or claim 3 wherein the surfactant is at least one non-ionic surfactant selected from the group consisting of the octylphenoxy polyoxyethanols (for example from the commercially available Triton ™ series), polyoxyethylene sorbitan esters (Tween ™ series) and polyoxythylene ethers or esters of general formula (I):

$$(I)\quad HO(CH_2CH_2O)_n\text{-}A\text{-}R$$

wherein n is 1-50, A is a bond or –C(O)-, R is $C_{1\text{-}50}$ alkyl or phenyl $C_{1\text{-}50}$ alkyl, and combinations of two or more of these.

5. The use according to claim 4 wherein the non-ionic surfactant is at least one surfactant selected from the group consisting of t-octylphenoxypolyethoxyethanol (Triton X-100), polyoxyethylene sorbitan monooleate (Tween 80) and laureth 9.

6. The use according to any one of claims 1 to 5 wherein the vaccine formulation contains a low dose of haemagglutinin.

7. The use according to claim 6 wherein the haemagglutinin content per influenza strain is about 30 μg or less.

8. The one-dose vaccine according to claim 7 wherein the haemagglutinin content per influenza strain is about 15 μg or less.

9. The use according to any one of claims 1 to 8 wherein the vaccine formulation is in a low volume per dose.

10. The use according to claim 9 wherein the volume per dose is less than 500 μl, or less than 300 μl or not more than about 200 μl per dose.

11. Use of a low dose of non-live influenza virus antigen material in the manufacture of an influenza vaccine for nasal administration.

12. The use according to claim 11 wherein the low antigen dose is about 30 μg or less of haemagglutinin per virus strain.

13. The use according to claim 12 in which the low antigen dose is about 15 μg or less of haemagglutinin per virus strain.

14. The use according to any one of claims 11 to 13 wherein the nasal vaccine is a one-dose vaccine.

15. The use according to any one of claims 1 to 14 wherein the vaccine is delivered in a bi-dose format of two sub-doses.

16. The use according to any one of claims 1 to 15, wherein the vaccine does not contain an added immunostimulant.

17. A method for prophylaxis of influenza infection or disease in a subject which method comprises administering to the subject a single dose of a non-live influenza virus vaccine via a mucosal surface.

18. A method for prophylaxis of influenza infection or disease in a subject which method comprises administering to the subject a low dose influenza preparation via a mucosal surface.

19. The method according to claim 17 or claim 18, wherein the vaccine is delivered intranasally.

~~20. A pharmaceutical kit comprising an intranasal delivery device and a one-dose~~ vaccine which comprises a non-live influenza virus antigen preparation without an added immunostimulant.

21. A pharmaceutical kit comprising an intranasal delivery device and a vaccine which comprises a low dose of a non-live influenza virus antigen preparation.

22. The pharmaceutical kit according to claim 20 or 21 wherein the device is a bi-dose delivery device for delivering two sub-doses in a single administration.

23. The pharmaceutical kit according to any one of claims 20 to 21 wherein the device is an intranasal spray device.

24. The use of a non-live influenza virus antigen preparation in the manufacture of a vaccine without any added immunostimulant for a one-dose vaccine against influenza.

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